APPLICATION UNDER UNITED STATES PATENT LAWS

Invention: USE OF FLP RECOMBINASE IN MICE

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This is a:

☐ Provisional Application

☒ Regular Utility Application

☐ Continuing Application

☐ PCT National Phase Application

☐ Design Application

☐ Reissue Application

☐ Plant Application

SPECIFICATION

USE OF FLP RECOMBINASE IN MICE

GOVERNMENT RIGHTS

BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention relates to the use of Flp recombinase to catalyze *FRT* site-specific DNA recombination in a transgenic non-human mammal, preferably a mouse.

2. Description of the Related Art

Site-specific recombinases are being developed as tools for genetic engineering because of their simplicity and precise activity in a variety of organisms. Two well studied recombinases are Flp and Cre. For use *in vivo*, a recombinase should be active in a transgenic non-human mammal. While Cre-mediated recombination has been successfully employed in trangenic mice, the utility of Flp recombinase in transgenic mice has not previously been established.

U.S. Pat. No. 4,959,317 discloses the use of the Cre-*lox* recombinase system in yeast and cultured mammalian cells, but not in transgenic mice. Other site-specific recombinases were not discussed.

U.S. Pat. No. 5,527,695 demonstrates the use of Flp recombinase in plants, but not in cultured mammalian cells or transgenic mice. A number of different site-specific

recombinase systems are discussed; however, no guidance appears to be given for selecting among the different systems and their use in a transgenic mouse is not discussed.

Kilby et al. (1993) reviewed the demonstrated activities of different site-specific recombinases in cells and organisms. Table 1 shows that, to their knowledge, Flp recombinase activity in transgenic mice had not been accomplished.

Flp-mediated deletion was demonstrated in embryonic stem (ES) cells by Jung et al. (1993). Gu et al. (1993) compared the activity of Cre and Flp recombinases in ES cells and found "a major fraction of ES cells transiently transfected by the *cre* vector undergo Cre-*loxP*-mediated gene deletion (which is not the case in our hands if the related FLP/FRT system from yeast is used [Jung et al., 1993, and unpublished data])". Both papers were contributed by the Rajewsky group, and the same group has exclusively used the Cre-*loxP* system in transgenic mice to inactivate endogenous genes, instead of Flp recombinase (Gu et al., 1994; Kühn et al., 1995). Thus, prior to the present invention, it was thought that using Cre recombinase was preferred over using Flp recombinase.

In view of the above teachings of the related art, it is an unexpected finding of the present invention that Flp recombinase can function in a developing mammal to catalyze *FRT* site-specific recombination.

Moreover, although Cre recombinase has been successfully used to create specific deletions in the mouse genome, the general utility of Cre to catalyze recombination is currently being established. Therefore, an additional method is needed for generating site-specific genetic alterations in the following ways: (1) a site-specific recombinase demonstrating a different dose-sensitivity could be used in situations where proper regulation of the recombination event cannot be achieved using Cre and (2) two site-

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specific recombinases could be used *in vivo* to engineer simultaneous or sequential

recombination reactions (e.g., independent gene activation or inactivation events).

For example, site-specific recombinases may be used to activate expression of a

tracer molecule to mark cell lineages. Factors that influence the determination of these

cell lineages can be identified by analyzing these marked cells in the genetic background

of various mutations, including mutations generated using the second recombinase system.

Additionally, having access to two recombinase systems allows for efficient use of the first

recombinase to generate a mutation, and the second recombinase to remove any selectable

markers used in generating that mutation which, if left in place, would confound

interpretation of the study. A second recombinase system is desired which exploits the

ability of Flp recombinase to catalyze FRT-specific recombination in a transgenic non-

human mammal which can be used alone or to expand the uses of the Cre/*loxP* system.

The present invention provides a transgenic non-human mammal with sufficient

Flp recombinase activity to catalyze recombination between *FRT* sequences, a transgenic

non-human mammal containing FRT target nucleic acid which serves as an efficient

substrate for Flp, a process of in vivo gene manipulation using the transgenic non-human

mammals, and a genetic system comprised of the Flp transgenic non-human mammal and

the FRT target transgenic non-human mammal which contains at least one *FRT* sequence.

BRIEF SUMMARY OF THE INVENTION

An object of the invention is to provide a transgenic non-human mammal with Flp

recombinase activity useful for manipulation of the genome in the intact mammal.

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Yet another object of the invention is to provide a process of *in vivo* genetic engineering using Flp recombinase activity to catalyze *FRT* site-specific recombination in a non-human mammal.

A further object of the invention is to provide a genetic system of the transgenic non-human mammal with Flp recombinase activity and at least one nucleic acid which is a substrate for Flp recombinase (e.g., the nucleic acid contains a *FRT* sequence). The nucleic acid may also contain a transgene for insertion into the genome of a non-human mammal, or a region which directs homologous recombination into the genome of a non-human mammal.

In one embodiment of the invention, a transgenic non-human mammal is provided which contains a Flp transgene integrated in its genome. Optionally, at least one Flp-recognition sequence is also integrated in the genome of the transgenic non-human mammal. The Flp-recognition sequence comprises *FRT* or a derivative thereof such as, for example, SEQ ID NO:14 or SEQ ID NO:15. A transgenic non-human mammal of the invention contains sufficient Flp recombinase activity in a cell to catalyze recombination between Flp-recognition sequences of the cell, chromosomal and/or extrachromosomal. Flp recombinase activity may be regulated by a chemical (e.g., exogenously administered drug, endogenous metabolite), the mammal's developmental stage, its body temperature, or tissue type of the cell.

The substrate for Flp recombinase activity is a Flp-recognition sequence. The genome of the transgenic non-human mammal may comprise one Flp-recognition sequence, two Flp-recognition sequences, or more than two Flp-recognition sequences. A chimeric or mosaic transgenic non-human mammal may contain cells with different

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numbers of Flp-recognition sequences due to Flp-mediated recombination; when a Flp-recognition sequence is integrated on only one of the pair of homologous chromosomes, the genome will be hemizygous for the Flp-recognition sequence.

Recombination between two Flp-recognition sequences integrated on different chromosomes results in translocation between those chromosomes. Such translocations are a common means of creating mutations that lead to developmental abnormalities or tumorigenesis.

Recombination between two Flp-recognition sequences in direct repeat orientation may cause excision of an intervening DNA sequence (e.g., a gene). Although such events are potentially reversible because Flp-mediated recombination is conservative, loss of the excised DNA sequence during cell division or by degradation makes the mutation irreversible. A null mutation in any gene may be created in this way, and the function of the gene studied in specific cells and/or at specific developmental stages.

Recombination between two Flp-recognition sequences in inverted repeat orientation may cause inversion of an intervening sequence or gene. As in *Salmonella* phase variation, inversion may cause activation or inactivation of a gene. If gene activity is detectable (e.g., selectable marker, histochemical marker, reporter gene), cell lineages may be traced by identifying recombination events that mark a cell and its descendants through detection of gene activation or inactivation. Cell lineages may be traced independent of gene activity, by monitoring differences in the integration site of the Flp substrate.

Recombination between a Flp-recognition sequence integrated on a chromosome and a Flp-recognition sequence integrated on extrachromosomal genetic material may

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cause insertion of the genetic material into the chromosome. An insertion created in this manner would provide means for creating transgenic non-human mammals with site-specific integration of a single copy of the transgene at a site in the genome specified by the chromosomal Flp-recognition sequence. Transgene insertion at a defined site in the genome would ensure reproducibility of expression because confounding effects of variable chromatin structure would be minimized.

Preferably, the intervening sequence or genetic material contains a gene such as, for example, a developmental gene, essential gene, cytokine gene, neurotransmitter gene, neurotransmitter receptor gene, oncogene, tumor suppressor gene, selectable marker, or histochemical marker, or portion thereof. Recombination may cause activation or inactivation of a gene by juxtaposition of regulatory regions to the gene or separation of regulatory regions from the gene, respectively.

The transgenic non-human mammal of the invention may also contain a Cre recombinase transgene. A cell of the transgenic non-human mammal would contain sufficient Cre recombinase activity to catalyze recombination between Cre-recognition sequences (e.g., *lox* site) of the cell.

A second embodiment of the invention is a process for *in vivo* genetic engineering using the transgenic non-human mammal. Flp recombinase activity is induced in a cell containing at least two Flp-recognition sequences at a level sufficient to catalyze site-specific recombination in the cell. This results in recombination between Flp-recognition sequences in the cell. The cell may be of germ line or somatic origin. If recombination occurs in a germ cell or a totipotent cell, offspring may be produced with a genome altered by the recombination event. A process for studying carcinogenesis and its treatment is

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provided by using Flp-mediated recombination to cause activation of an oncogene or inactivation of a tumor suppressor gene. Candidate compounds or compositions may be screened in such a process to identify candidates that act to promote carcinogenesis (i.e., a cancer promoter) or inhibit carcinogenesis (i.e., a cancer inhibitor). Similarly, Flp-mediated recombination in a transgenic non-human mammal may be used in a process of activating ectopic expression of a gene during development, inactivating expression of a gene at a specific time or in a specific tissue, or identifying a cell lineage by activation or inactivation of a gene. The gene may be a developmental gene, essential gene, cytokine gene, neurotransmitter gene, neurotransmitter receptor gene, oncogene, tumor suppressor gene, selectable marker, or histochemical marker.

In a third embodiment of the invention, a genetic system comprising the transgenic non-human mammal and a purified nucleic acid containing at least one Flp-recognition sequence is provided. Preferred nucleic acids include the vectors described herein. Optionally, the genetic system may also comprise means for producing a transgenic non-human mammal which contains at least some portion of the purified nucleic acid; preferably, at least the Flp-recognition sequence is integrated into the genome of the transgenic non-human mammal.

As used herein, a transgenic non-human mammal is a non-human mammal into which genetic material has been introduced with a recombinant nucleic acid. The introduced genetic material may become integrated into the genome of the non-human mammal, preferably stably- or excisably-integrated into a chromosome of the non-human mammal, and be transmitted through the germ line to a succeeding generation.

Alternatively, the genetic material may be maintained as an episome or an artificial chromosome.

Any such introduced genetic material is termed a transgene. If the transgene is unstable or is excised during cell division, the result will be a mosaic mammal comprised of at least two cell types with different genetic content but derived from the same zygote. Such an mammal may be used in cell lineage tracing; thus, a transgene flanked by *FRT* sites may be excised during ontogeny by the action of Flp recombinase. In contrast, a chimeric mammal comprises at least two genetically different cell types derived from different zygotes (e.g., a mammal resulting from injection of embryonal carcinoma or embryonic stem cells into a genetically different blastocyst). If the transgenic non-human mammal only contains the transgene in somatic cells, the transgene will not be passed to a succeeding generation through the germ line.

Preferably, the non-human mammal is a mammal for which methods to introduce a transgene are known in the art such as, for example, cow, goat, mouse, pig, rabbit, rat and sheep. Such methods of introducing genetic material include microinjection for the creation of transgenics from zygotes; and electroporation, biolistics, lipofection, calcium phosphate-DNA co-precipitation, DEAE-dextran, microinjection, and viral infection for the creation of transgenics from a cultured cell (e.g., pluripotent cells such as a teratocarcinoma or embryonal carcinoma, totipotent cells such as an embryonic stem cell) and subsequent transfer into an embryo.

The transgenic non-human mammal, the process, and the genetic system are particularly advantageous when separate control of more than one recombination event is desired in the mammalian genome. For example, integration of a *loxP* sequence-

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containing substrate may be followed by deletion of a selectable marker from the substrate which is mediated by Flp recombinase activity catalyzing recombination between *FRT* sequences flanking the selectable marker, and Cre-mediated recombination of the *loxP*-containing substrate. Integration of the *loxP* sequence containing substrate is preferably by homologous recombination, and expression of the co-integrated selectable marker enriches for this rare event; the second event occurs by Flp-mediated site-specific recombination which deletes the selectable marker to reduce competition from the regulatory regions of the selectable marker; and the third event of deleting gene sequences lying between *loxP* sites occurs by Cre-mediated site-specific recombination.

DESCRIPTION OF THE DRAWINGS

Figure 1. Structure of the modular fusion vectors. Each functional segment is represented by a patterned rectangle (not drawn to scale); the name of each segment is boxed and described below. Enzymes in parentheses cut twice in the vectors (except plasmid $pFRT_2$ which contains three *Xba*I sites), all other enzymes shown are unique. A unique *Cla*I site at the junction of *trpS* and *lacZ* sequences is not diagrammed because it is resistant to cleavage when grown on Dam^+ *E. coli* strains. All vectors, except $pFRT_2$, are built on a pUC19-derived plasmid backbone containing Ap^R (Yanisch-Perron et al., 1985; Fire et al., 1990). Plasmid $pFRT_2$ is built on a pCR backbone containing both Ap^R and Km^R (Invitrogen). Complete sequences for all vectors are available from GenBank (accession numbers U46489-U46493). (Fig. 1A) Structure of the *Flp*-expression vector. To generate pFlp, sequences were removed from p16.43 (Fire et al., 1990) and replaced with a *Xba*I-*Sal*I fragment containing the synthetic intron, *Flp*, and SV40 p(A) from

pOG44 (O'Gorman et al., 1991). (Fig. 1B) Structure of the *lacZ* fusion vector placZ.

(Fig. 1C) Structure of the target *FRT* vectors. The *FRT*-cassette was constructed in two

steps. Double-stranded oligos (oligo pairs SD1/SD3 and SD2/SD4) were ligated and then

amplified by PCR using oligo pair SD9/SD10. The 150-bp PCR product bracketed by

*Age*I sites was then inserted into pCR (Invitrogen) to generate pFRT$_2$. Oligos used to

construct the *FRT$_2$-Age*I cassette include SD1 (SEQ ID NO:1), SD2 (SEQ ID NO:2), SD3

(SEQ ID NO:3), SD4 (SEQ ID NO:4), SD9 (SEQ ID NO:5), and SD10 (SEQ ID NO:6).

Plasmid pFRT$_2$lacZ was generated by inserting the *FRT$_2$-Age*I fragment from

pFRT$_2$ into the unique *Age*I site in placZ. This insertion disrupts the *NLS::trpS::lacZ* open

reading frame; there are no ATG codons downstream of the *Age*I site to start translation of

functional ßGal. Plasmid pFRT$_2$neo.lacZ contains the Nm^R gene (*Xho*I-*Bam*HI fragment)

from pNeoßGal (O'Gorman et al., 1991) inserted between the *Bgl*II and *Xho*I sites of

pFRT$_2$lacZ. The orientation of *neo^R* is opposite to *lacZ*. Plasmid pFRT.lacZ was

generated by digesting pFRT$_2$lacZ with *Xba*I and religating. Corresponding *FRT* vectors

have been generated which contain a SV40 small *t* intron immediately 5' to the p(A)

(*Xba*I-*Not*I fragment from pEµSv; Rosenbaum et al., 1989). These vectors are designated

the "t" series (placZ.t, pFRT$_2$lacZ.t, pFRT$_2$neo.lacZ.t and pFRT.lacZ.t). "FRT$_2$" defines

two target sites in the same orientation; "FRT" refers to a single target site. Abbreviations

used for restriction enzymes are as follows: *Age*I (A); *Apa*I (Ap); *Avr*IIc (Av); *Bam*HI

(B); *Bgl*II (Bg); *Bss*HII (Bs); *Bst*BI (Bt); BstXI (Bx); *Eag*I (E); *Eco*RI (RI); *Eco*RV (RV);

*Hind*III (H); *Kpn*I (K); *Nhe*I (Nh); *Not*I (N); *Pst*I (P); *Sac*I (Sa); *Sal*I (S); *Sma*I (Sm);

*Sna*BI (Sn); *Spe*I (Sp); *Sph*I (Sh); *Sse*8387I (Ss); *Stu*I (St); *Tth*111I (T); *Xba*I (X); *Xca*I

(Xc); *Xho*I (Xh).

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Figure 2. Schematic and partial sequence of the ßGal fusion protein predicted to be encoded by the product of Flp-mediated recombination (the nucleotide sequence shown is SEQ ID NO:7 and the protein sequence shown is SEQ ID NO:8). Linked in order are the initiating ATG codon, the 8-aa SV40 T antigen nuclear localization signal (NLS, SEQ ID NO:9), the 16-aa domain encoded by the residual FRT (SEQ ID NO:10), the 27-aa TrpS linker (SEQ ID NO:11), and ßGal. The FRT consists of two 13-bp inverted repeats (SEQ ID NOS:12-13) flanking an 8-bp spacer that comprise the minimal Flp-recognition sequence (SEQ ID NO:14), plus an additional 13-bp repeat (SEQ ID NO:15).

Figure 3. DNA constructs and the Flp-mediated recombination event. Structure of target and recombinase transgenes. Flp recombination targets (FRTs) are depicted as black triangles. Rectangles represent exons; heavy lines, introns and flanking regulatory sequences; thin lines, vector sequences; and small arrows, translation start sites. Hybridization probes are represented by numbered lines. PCR oligonucleotide primers are represented by small half arrows. (Fig. 3A) Structure of target transgenes. Plasmid pFRTZ (for FRT-disrupted lacZ transgene) contains 3.9-kb of sequence from the human β-actin (hACTB) gene (Gunning et al., 1987; Zhang et al., 1994) inserted into the target vector pFRT₂neo.lacZ; a nuclear localization signal (NLS) and SV40 early polyadenylation (pA) sequence are also included. pFRTZ.2 is an alternative target plasmid which contains the HSV-tk gene inserted between the FRT sequences of pFRTZ. Control plasmid pFRTZ-product represents the product of Flp-mediated excisional recombination. Restriction sites and probe 1 used in Southern analyses are shown on pFRTZ. (Fig. 3B) Structure of FLP transgenes. Plasmid phACTB::FLP contains the 3.9-kb hACTB fragment inserted into the expression vector pFLP, which contains a synthetic

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intron, Flp-encoding sequence, and SV40 late pA sequence from pOG44 (O'Gorman et al.,

1991; Stratagene). pRevhACTB::FLP contains the h*ACTB* sequences in reverse

orientation and serves as a negative control. Plasmid pWnt1::FLP contains the synthetic

intron, Flp-encoding sequence and the SV40 late pA from pFLP inserted into the

polylinker of the *Wnt-1* expression vector pWEXP2 (26). Probe 2 is used in the whole

mount *in situ* hybridization analyses; probe 3 is used in the Northern analyses. (Fig. 3C)

Diagram of the FLP-mediated excisional recombination reaction.

DETAILED DESCRIPTION OF THE INVENTION

Site-specific recombinases of the integrase family, including Flp, are being

developed as tools for genetic engineering because of their simplicity and precise activity

in a broad range of organisms (Broach et al., 1982; Cox, 1983; Vetter et al., 1983; Stark et

al., 1992; Kilby et al., 1993; Snaith et al., 1995). Flp recombinase from the yeast

Saccharomyces cerevisiae can catalyze excision, insertion, inversion, and translocations of

DNA containing Flp-recognition target sites without other cofactors (Cox, 1983; Vetter et

al., 1983). The type of recombination product is determined by the orientation of target

sites relative to each other on a segment of DNA (Stark et al., 1992; Kilby et al., 1993):

directly repeated sites specify excision of intervening DNA, inverted sites specify

inversion of intervening DNA, and sites on separate DNA molecules specify either

translocation, insertion, or duplication (a special case of insertion with identical DNA

molecules). Controlled recombinase expression in an organism bearing chromosomal

recombinase target sites can be used to generate tissue-specific mutations, or to assess the

effect of ectopic gene expression in a subset of cells with an otherwise normal organism.

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These applications are particularly desirable when studying the effect of lethal or otherwise deleterious mutations, or when null mutations of a gene do not result in an observable phenotype. Such controlled gene expression and manipulation can also be used to activate or inactivate a lineage tracer in populations of cells and their descendants, so that cell lineages can be identified and functionally characterized. Together, these applications provide a means to address many previously intractable problems in mammalian development.

Flp recombinase recognizes a distinct 34-bp minimal site which tolerates only limited degeneracy of its recognition sequence (Jayaram, 1985; Senecoff et al., 1988). In site-specific recombination, the substrate DNA is broken at two points and the ends are exchanged, with no synthesis or degradation of DNA (i.e., conservative recombination). For example, recombination with a circular DNA substrate can have three outcomes: a single circle resolved into two circles, two circles fused into one, or a segment within a circle can be inverted with respect to the rest of the circular molecule. To achieve a precise joint in the recombined product, the recombinase must locate the sites for cutting and rejoining the substrate, bring them together, and catalyze digestion, exchange and ligation of the substrate in a well-regulated manner. The top and bottom strand cuts are at fixed points within the crossover region but staggered, such that cleavage on both strands would give recessed 3' ends.

Controlled recombinase expression in a transgenic non-human mammal carrying appropriately placed target sites in its genome can be exploited to alter the genotype of a subset of cells within an otherwise normal embryo or adult. Such mosaic mammals bearing clones of genetically distinct somatic cells have been most extensively generated

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in *Drosophila*, providing new means to address previously intractable problems. For example, Flp-mediated excisional recombination may be used to irreversibly activate or inactivate a marker gene in specific cell populations and their descendants, allowing cell lineages to be studied; similarly, genes of interest may be ectopically expressed to study their effects on differentiation and development. By promoting mitotic exchange between target sites on homologous chromosomes, Flp provides an effective methodology for F_1 genetic screens. By catalyzing recombination between target sites on the same DNA molecule or between targets sites on different DNA molecules, the Flp site-specific recombinase can be used to study a variety of biological processes. Importantly, such recombination schemes can be used to generate tissue- or stage-specific mutations that would be lethal if generated in the whole organism. Controlled Flp recombinase expression, in an organism carrying chromosomal Flp recombinase target (*FRT*) sites, can be used to generate tissue- or stage-specific mutations, or to assess the effects of transgene expression in a subset of cells within an otherwise normal mammal.

In the examples below, the nucleotide sequence of the Flp gene used was SEQ ID NO:16 and the Flp protein had amino acid sequence SEQ ID NO:17. The wild-type Flp gene from Saccharomyces cerevisiae has nucleotide sequence SEQ ID NO:18 which encodes the amino acid sequence SEQ ID NO:19.

The wild-type Flp recombinase is most active at 23-30°C and loses activity at higher temperatures. The Flp recombinase used in the examples below include the F70L mutation which has the characteristics of a temperature-sensitive mutation with activity between 23-30°C equivalent to that of wild-type Flp recombinase, but little activity at 37°C and above (Buchholz et al., 1996). This led to the recommendation by Stewart's

group that Cre recombinase be used rather than Flp recombinase in applications that require "quantitative recombination". In contrast, the present invention illustrates the utility of non-human mammals containing Flp transgenes.

By use of the degenerate genetic code, variant nucleotide sequences may be generated that are translated into SEQ ID NO:16 and, thus, encode Flp protein. Variant nucleotide sequences may be selected for translation by considering the frequency of codon usage in the non-human mammal. Functional equivalents of the disclosed nucleotide sequence may be generated by making minor sequence variations in SEQ ID NO:15 and measuring recombinase activity of the translated variant protein. A functional equivalent of Flp recombinase would mediate site-specific recombination more efficiently than random recombination, and produce recombined *FRT* sequence joints by precise cleavage and ligation. The degree of sequence variation may be assessed by low or high stringency hybridization with a target sequence of SEQ ID NO:15, or by reference to substitution of codons for chemically similar amino acid residues. Examples of Flp variants are given in Kulpa et al., (1993). Variant proteins may also be selected with desired properties such as thermolability, thermostability, altered cellular localization, altered sequence recognition, and/or modified frequency of recombination. For example, a Flp variant comprising a nuclear localization signal and Flp recombinase as a fusion protein may be used to target Flp recombinase to the nucleus, and thereby increase the frequency of recombination.

Flp recombinase activity may have its expression controlled by a regulatory region operably linked to a Flp transgene. By appropriate choice of regulatory region, Flp recombinase activity may be regulated by a chemical (e.g., drug administered to the

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mammal, endogenous metabolite of the mammal), developmental stage, temperature, type

of tissue or cell of origin, or a combination thereof. The regulatory region may express the

transgene in a broad (e.g., ubiquitously) or narrow (e.g., tissue specific) range of tissues, at

varying levels of expression which may lead to different frequencies of recombination, or

in a sustained or transient manner. Similar regulatory regions may be operably linked to a

developmental gene, essential gene, cytokine gene, neurotransmitter, neurotransmitter

receptor, oncogene, tumor suppressor gene, selectable marker, or histochemical marker to

confer desired expression on the gene or marker; such gene or marker may be genetically

manipulated *in vivo* by linkage to a Flp-recognition sequence and the action of Flp-

mediated recombination.

Regulatory regions may control transcription (e.g., promoter, enhancer, silencer,

rate of elongation), post-transcriptional events (e.g., editing, splicing, message stability,

polyadenylation, transport out of the nucleus), translation (e.g., initiation, elongation,

termination), or post-translational events (e.g., secretion and transport, cell localization,

folding, assembly, protease cleavage or degradation, acylation, glycosylation, sulfation,

phosphorylation, isomerization). Transcription may be regulated by, for example,

tetracyline, bacteriophage RNA polymerase, IPTG, heavy metal, steroid, viral infection,

expression of a DNA-binding factor, modulation of a DNA-binding factor by chemical

inducers of dimerization, developmental stage, heat, tissue type, or a combination thereof.

Regulatory regions may be selected from genes such as, for example, metallothionein, heat

shock protein, globin, immunoglobulin heavy chain, lens crystallin, muscle creatine

kinase, elastase, enolase, serum-responsive genes, major histocompatibility complex class

II, interferon-responsive genes, steroid-responsive genes or the like. Regulatory regions

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for ubiquitous expression may be selected from genes such as, for example, β-actin,

phosphoglycerate kinase, HMG-CoA reductase, major histocompatibility complex class I,

β2-microglobulin, HSV thymidine kinase gene, Rous Sarcoma Virus regulatory elements,

CMV immediate-early gene, SV40 origin, or the like.

Examples of regulatory regions and useful methods of gene expression are given in

Goeddel (1990), Kriegler (1990), and Murray (1991). U.S. Pat. No. 5,221,778 describes a

binary transgenic system comprised of a line with a transactivator-dependent promoter

controlling expression of a transgene crossed to a line expressing the transactivator

ubiquitously, at specific times, or in specific cells. Such a system may be used to regulate

the Flp recombinase transgene in the parent or in offspring.

Examples of developmental genes that may be studied by Flp-mediated

recombination include: adhesion molecules (e.g., N-CAM, cadherins, integrins), cyclin

kinase inhibitors (e.g., p27, p21, p16, p15), Wnt family members, Pax family members,

Winged helix family members, Hox family members, cytokines, interleukins,

growth/differentiation factors (e.g., BMPs, EGF, netrin, PDGF, TGF-β) and their

receptors, neurotransmitters and their receptors, kinase and phosphatases, metabolic

enzymes such as tyrosine hydroxylase, antigen receptors (TCR and Ig). The gene may be

expressed in a temporal and spatial pattern that mimics endogenous gene regulation to

rescue lethal mutations, or otherwise complement mutant genes. While expressing the

developmental gene in cells responsible for the lethal phenotype, the function of the gene

may be determined in other cell types by selectively inactivating gene expression in those

other cells. The function of a gene may also be determined by over expressing the gene.

A developmental gene may also be expressed ectopically, that is expressed in a temporal

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or spatial pattern that does not mimic the endogenous patterns of expression. Thus, the function of the gene may be determined at a stage of the cell or in a type of cell with a different developmental program than is normally encountered through inactivation, over expression, or ectopic expression.

A series of recombination events may be controlled by the use of multiple Flp-recognition sites, or by the use of different site-specific recombination systems. Control of a series of recombination events may be accomplished by a competition for Flp recombinase by a multitude of identical Flp-recognition sites; recombination between identical Flp-recognition sites will then occur stochastically because the Flp-recognition sites may be used as substrates of Flp recombinase at equivalent frequencies. The physicial contacts between Flp recombinase and a *FRT* sequence have been examined (Panigrahi et al., 1992). Alternatively, derivatives of the *FRT* sequence may be used as Flp-recognition sites: if the derivative is used as a substrate of Flp recombinase with lower efficiency, then Flp-mediated recombination at that site will occur less frequently; or if the derivative is used as a substrate of Flp recombinase with higher efficiency, then Flp-mediated recombination at that site will occur more frequently. Recombination between two Flp-recognition sequences results in a hybrid Flp-recognition sequence (i.e., a 13-bp repeat from a first Flp-recognition sequence and a 13-bp repeat from a second Flp-recognition sequence); thus, two barely functional *FRT* derivatives, each with a mutation in a 13-bp repeat (termed a half site), will exchange half sites after Flp-mediated recombination and may result in one functional *FRT* and one non-functional *FRT*. Examples of variant *FRT* sequences are given by Jayaram (1985) and Senecoff et al. (1988), and an assay for Flp-mediated recombination on different substrates is described

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by Snaith et al. (1996). Besides sequence diversity, the efficiency of Flp recombinase for a given substrate may be determined by local copy number of Flp-recognition sequences, or chromatin structure.

Recombination frequency may also be controlled by using derivatives of Flp recombinase which may have altered functions such as binding of the recognition sequence, cleavage of DNA, and rejoining of DNA. Fusion proteins comprising a Flp recombinase domain may be engineered to make recombinase activity of the fusion protein dependent on the availability of a diffusible ligand and binding of the ligand to the fusion protein (Logie and Stewart, 1995). A Flp transgene may be expressed under the control of a regulatory region that confers stage- or tissue-specific expression as described hereinabove. Flp recombinase may be expressed as part of a binary system, i.e. the Flp transgene may be operably linked to a regulatory region which is responsive to a transcription factor and the transcription factor is produced by another transgene which is integrated at a site not linked to the integration site of the Flp transgene. Preferably, the Flp transgene is regulated by a substance that is not naturally found in the transgenic host such as, for example, tetracycline (U.S. Pat. No. 5,589,362), cyclophilin (Belshaw et al., 1996), ecdysone (No et al., 1996), IPTG (U.S. Pat. Nos. 4,833,080 and 5,589,392), T7 RNA polymerase (U.S. Pat. No. 5,550,035). An alternative to controlling recombinase activity is to separate the recognition and catalytic domains of the Flp recombinase into separate polypeptides that are non-functional on their own, but that are brought together to form a functional recombinase when a diffusible binding molecule is administered to the transgenic mouse, to multimerize Flp recombinase monomers, to translocate Flp recombinase into the nucleus, or to recruit transcriptional activators that control expression

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of the Flp recombinase transgene (e.g., chemical inducer of dimerization; Belshaw et al., 1996; Crabtree and Schreiber, 1996); or to engineer a fusion protein of Flp and a ligand-binding domain (Logie and Stewart, 1995).

Moreover, a distinct site-specific recombination system may be used (e.g., Cre-*lox*) to differentially control a series of recombination events by expressing Flp and Cre recombinases independently of each other.

A genetic system with a first line of non-human mammal with a Flp transgene and a second line of transgenic non-human mammal with a transgene containing at least one Flp-recognition site is another way of regulating Flp-mediated recombination. By breeding the two transgenic lines, the Flp transgene and its substrate will be present and Flp-mediated recombination will occur in cells with sufficient Flp recombinase activity and access to substrate.

In a mosaic or a chimeric transgenic non-human mammal, cells that have undergone site-specific recombination between Flp-recognition sequences may contain different numbers of Flp-recognition sequences. In mosaic or chimeric transgenic non-human mammals that have undergone Flp-mediated inversion of a second transgene flanked by Flp-recognition sequences in inverted repeat orientation, cell lineages may be traced by activation (e.g., transcription of the sense strand by a juxtaposed regulatory region) or inactivation (e.g., deletion or separation from a regulatory region) of the second transgene. The second transgene is preferably a histochemical marker for tracing cell lineages, but *in situ* hybridization of transcripts from the second transgene is a possible method of detecting gene expression. Histochemical markers or reporter genes include alkaline phosphatase, β-galactosidase, choramphenicol acetyltransferase, luciferase, green

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fluorescent protein, β-glucoronidase, or derivatives thereof (Wasserman and DePamphilis, 1993). For *in situ* hybridization, radioactive labels may be used, but non-radioactive methods using an enzymatic label as described above and avidin-biotin or digoxygenin-antibody interaction. Another way of assaying gene activity would be to tag the marker gene with a peptide sequence (i.e., a fusion protein) that is capable of specific binding, and to detect the presence of the fusion protein with a cognate binder of the peptide tag. Useful peptide tag-binder pairs include, but are not limited to, avidin-biotin, GST-glutathione, polyHis-divalent metal, MBP-maltose, 9E10 Myc epitope-antibody, protein A/G-immunoglobulin, and SV40 T antigen-antibody.

Fiering et al. (1995) have described a method for gene replacement by homologous recombination, followed by site-specific recombination in ES cells to delete a selectable marker. First, the gene is replaced by homologous recombination in ES cells with positive (*neo*) - negative (HSV *tk*) selection. Homologous recombination results in retention of the *neo* selectable marker and loss of the HSV *tk* selectable marker at the homologously recombined locus, but removal of the remaining selectable marker is essential to eliminate competition between endogenous promoters and the promoter of the selectable marker. The *neo* expression cassette was deleted by Flp-mediated recombination in ES cells, but Fiering et al. did not express Flp recombinase in a transgenic mouse. Examples of selectable markers include neomycin phosphotransferase, hygromycin phosphotransferase, puromycin N-acetyl transferase, dihydrofolate reductase, HSV thymidine kinase, adenosine deaminase, hypoxanthine-guanine phosphoribosyl transferase, and adenine phosphoribosyl transferase. Cells carrying the marker may be selected through use of an exogenously added drug or an endogenous metabolite.

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The process of screening candidate drugs that affect tumorigenesis will be directed to particular oncogenes or tumor suppressor genes. By controlling Flp-mediated recombination, such genes can be activated, inactivated, or otherwise mutated in a tissue- or temporal-specific manner. For example, MYC recombination may be directed to lymphocytes by expressing Flp recombinase with a lymphocyte-specific regulatory region from immunoglobulin heavy chain (IGH). A preferred means of activating MYC would be translocation to the IGH locus as occurs in murine plasmacytoma or human Burkitt lymphoma. A tumor suppressor gene such as RB1 could be inactivated by deletion or point mutation by expressing Flp recombinase with a crystallin regulatory region. In the foregoing, by controlling activation (MYC) or inactivation (RB1) in the cellular compartment (lymphocyte or lens, respectively) in which the gene functions, the model recapitulates a natural process of tumorigenesis.

Control of different recombination events would be useful in investigating models of multi-hit carcinogenesis, or in determining whether an immortalized or transformed phenotype can be reversed by inactivation of an oncogene (Westerman and Leboulch, 1996) or activation of a tumor suppressor gene.

A non-limiting list of oncogenes includes ABL1, BCL1, BCL2, BCL6, CBFA2, CBL, CSF1R, ERBA, ERBB, EBRB2, ETS1, ETV6, FGR, FOS, FYN, HCR, HRAS, JUN, KRAS, LCK, LYN, MDM2, MLL, MYB, MYC, MYCL1, MYCN, NRAS, PIM1, PML, RET, SRC, TAL1, TCL3, and YES. The Flp recombinase system may be used to mutate the oncogene or its homologs by genetic means such as, for example, excision, integration (which may lead to duplication of the gene), inversion, translocation, or a combination thereof; types of mutation include amplification, deletion, gain of function,

insertion, loss of expression, loss of function, over expression, or a combination thereof. Some oncogenes, especially those that encode transcription factors, are mutated by chromosome translocation or inversion; regulatory regions may be juxtaposed to the coding region of the oncogene to cause aberrant expression, or a fusion protein may be formed from all or part of the oncogene (reviewed by Rabbitts, 1994; Sorensen and Triche, 1996). Other oncogenes are often activated by point mutations that have functional consequences (e.g., gain of function, loss of function) upon translation of the oncoprotein. Oncogenes may be over expressed by gene amplification, or activation by regulatory regions that are juxtaposed by recombination next to the oncogene. Tumors may be of hematologic origin (e.g., leukemia, lymphoma) or a solid tumor.

Tumor suppressor genes include, but are not limited to, APC, BRCA1, BRCA2, MADH4, MCC, NF1, NF2, RB1, WT1, and TP53. Defined deletions of the tumor suppressor gene or its homologs may be made in whole, or in part, by Flp-mediated recombination. Flp recombinase may mutate the tumor suppressor gene or its homologs by the means previously described for oncogenes, or may induce a loss of heterozygosity by inactivating a functional tumor suppressor gene or its homologs which is allelic to a recessive mutation. The gene sequence may encode the tumor suppressor gene or its homologs, or may contain a region that regulates expression of the tumor suppressor or its homologs at the level of, for example, transcription or translation. Flp recombinase activity may induce a chromosome translocation which disrupts the coding sequence of the tumor suppressor gene or its homologs, or separates the coding sequence from essential regulatory regions. Small intragenic or large chromosome inversions may serve the same purpose.

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The above-mentioned names follow the genetic nomenclature of Online Mendelian Inheritance in Man (May 1997), Center for Medical Genetics, Johns Hopkins University and National Center for Biotechnology Information, National Library of Medicine. Either the human gene or its homolog may be introduced into the non-human mammal as a transgene, or an endogenous homolog of the human gene may be genetically manipulated in the non-human mammal. Such manipulation may include introduction of one or more FRT sequences within or nearby the homologous gene by means known in the art.

All books, articles and patents cited in this specification are incorporated herein by reference in their entirety.

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The following examples are meant to be illustrative of the present invention; however, the practice of the invention is not limited or restricted in any way by them.

EXAMPLE 1

Flp Recombinase and Target *FRT* Vectors

A set of versatile Flp recombinase and target vectors was constructed to facilitate using the Flp recombinase to manipulate DNA in cell culture and in the whole animal. These vectors are described below and shown in Fig. 1.

pFlp (SEQ ID NO:15), a vector for directed expression of Flp recombinase: The pFlp plasmid contains unique 5' and 3' polylinker segments (multiple cloning sites, MCS), to facilitate insertion of regulatory elements to direct recombinase expression (Fig. 1A). This plasmid consists of a 5' MCS, Flp coding sequence (O'Gorman et al., 1991), SV40 late orientation p(A) sequence and a 3' MCS. A synthetic intron (Huang and Gorman, 1990; O'Gorman et al., 1991) has been placed upstream of the Flp coding region to maximize expression.

placZ, a lacZ fusion vector for use in vertebrate cells: Rapid and precise histochemical staining procedures make ßGal a good reporter for assays of gene expression in transgenic animals. To facilitate construction of *lacZ* fusions for expression in vertebrate cells, the plasmid placZ has been generated. It is comprised of a 5' MCS , a

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*Kpn*I-cassette containing a start Met codon and a nuclear localization signal (NLS), the ßGal-coding region, SV40 early p(A) sequence and a 3' MCS (Fig.1B).

The *Kpn*I-cassette in placZ contains an ATG within an optimized vertebrate translation start sequence (Kozak, 1987), followed by sequences in frame that encode the NLS of SV40 T antigen (Kalderon et al., 1984a). For transcriptional fusions where translation starts at this ATG, the NLS will reside on the N-terminus of ßGal and can confer tight nuclear localization upon ßGal (Kalderon et al., 1984b; Bonneret et al., 1987). Nuclear-localized ßGal, in contrast to cytoplasmic ßGal, can help identify marked cells in the context of complex tissues and can readily be distinguished from any endogenous cytoplasmic ßGal activity. Nuclear localization of ßGal also permits simultaneous immunodetection of cytoplasmic cell identity markers allowing for unambiguous phenotyping of ßGal marked cells. If the translation start signal and NLS are not desired, the cassette can be removed by cutting with *Kpn*I (or *Asp718*I).

The ßGal-coding region was derived from an *E. coli trpS::lacZ* fusion (Hall et al., 1983; Fire et al., 1990). Consequently, the *lacZ*-containing vectors encode fusion proteins with a short peptide leader from TrpS attached to the N-terminus of ßGal.

Target FRT vectors: Recombinase target vectors have been generated that allow specific combinations of *FRT*s, signals for RNA processing and protein targeting, selection markers, and reporter genes (or other DNA sequences) to be linked as needed (Fig. 1C).

The '*FRT₂-Age*I cassette' vector (pFRT₂) contains two recombination target sites in direct orientation flanked by *Age*I sites. Each target site consists of two 13-bp inverted repeats flanking an 8-bp spacer (the minimal Flp-recognition site), plus an additional 13-

bp repeat that can enhance substrate activity (Jayaram, 1985). The nucleotide sequence of a single *FRT* target site is shown in Fig. 2. In plasmid pFRT$_2$ a MCS has been engineered between the *FRT* sites to allow insertion of various DNA sequences.

The *FRT*-disrupted (silent) *lacZ* fusion vector, pFRT$_2$lacZ, was designed to optimize expression of *lacZ* and detection of ßGal following Flp-mediated excisional recombination. It was generated by inserting the FRT$_2$-*Age*I fragment from pFRT$_2$ into the unique *Age*I site in placZ. This *Age*I-insertion disrupts the *NLS::trpS::lacZ* open reading frame. Because there are no ATG codons to start translation of functional ßGal downstream of the *Age*I site, ßGal activity is strictly dependent on Flp-mediated excisional recombination. Following Flp-recombination, pFRT$_2$lacZ encodes a fusion protein containing an 8-aa NLS, a 16-aa domain encoded by the residual *FRT*, and a 26-aa peptide leader from TrpS attached to the N-terminus of ßGal (Fig. 2). This ßGal fusion protein generates robust enzyme activity in mammalian cells.

Plasmid pFRT$_2$neo.lacZ contains the Nm^R gene, *MC1::neo^R::pA* (O'Gorman et al., 1991), inserted between the *FRT*s of pFRT$_2$lacZ. The *neo^R* gene enables selection of transgene integrants in cell culture. Plasmid pFRT.lacZ represents the expected product of Flp recombination and serves as a useful 'pre-activated' control.

To enhance transcript processing, stability and translation in mammalian cells (Brinster et al., 1988; Buchman and Berg, 1988; Huang and Gorman, 1990), a set of plasmids has been constructed that contain the SV40 small *t* intron immediately 5' to the p(A). The modified vectors comprise the "t" series (pFRT$_2$lacZ.t, pFRT$_2$neo.lacZ.t, pFRT.lacZ.t and placZ.t).

Considerations When Building Transcriptional Fusions

Transcriptional fusions use regulatory regions from the gene of interest linked so that translation starts at the ATG within the *KpnI-NLS*-cassette. When designing such fusions it is important that the initiating ATG of NLS be the first ATG codon in the mRNA. The ATG in the *SphI* site in the 5' MCS therefore precludes use of the *Hind*III and *SphI* sites as right-hand junctions in transcriptional fusions (Fire et al., 1990). If chosen regulatory regions contain introns, the transcriptional fusion should not be constructed in the "t" vectors. The presence of the SV40 small *t* intron 3' to *lacZ* may result in aberrant splicing if additional introns are fused 5'.

Flp-Dependent ßGal Activity in Mammalian Cell Culture

The integrity of the Flp, *FRT* and *lacZ* vectors has been confirmed by restriction mapping, nucleotide sequencing and demonstration of Flp-dependent ßGal activity following cotransfection of embryonic fibroblasts. For the cotransfection assays, regulatory sequences from the human ß-actin-encoding gene (h*ACTB*) were used to direct transgene expression (Gunning et al., 1987; Zhang et al., 1994). A 3.9-kb sequence from h*ACTB* (3-kb 5' flank, 78-bp 5' untranslated region, and 832-bp first intron) was inserted into the 5' MCS sites of pFlp, pFRT₂neo.lacZ, pFRT.lacZ and placZ to generate phACTB::Flp, phACTB::FRT₂neo.lacZ, phACTB::FRT.lacZ and phACTB::lacZ, respectively.

In transient transfection experiments using primary embryonic fibroblasts (Robertson, 1987), robust nuclear ßGal activity was observed by XGal histochemical staining (Sanes et al., 1986) two days following calcium phosphate transfection (Gorman

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et al., 1982) with the "pre-activated" constructs phACTB::FRT.lacZ and phACTB::lacZ. In contrast, ßGal activity from the phACTB::FRT$_2$neo.lacZ construct was observed only following cotransfection with phACTB::Flp. Plasmid phACTB::FRT$_2$neo.lacZ transfected alone, or with *Flp* driven by the h*ACTB* promoter in the reverse orientation, showed no detectable ßGal activity.

Conclusions

(1) Flp recombinase and *FRT* target vectors have been generated. When fused to h*ACTB* regulatory sequences, these vectors have been shown to function in mammalian cells with ßGal activity strictly dependent on co-expression of Flp. The SV40 NLS fused to the *FRT*-encoded domain (the residual *FRT* following recombination) can confer nuclear localization on ßGal in mammalian cells. Having validated these vectors in cell culture, they are used below to establish a histochemical marking system in the mouse to study cell lineage.

(2) Because *lacZ* can be activated by Flp-mediated excisional recombination, these vectors would be expected to enable activation or excision of any transgene of interest.

(3) The unique restriction sites in pFlp facilitate insertion of a variety of regulatory elements to direct Flp expression, and therefore recombination, to specific cells or tissues of a transgenic non-human mammal.

(4) By coupling tissue-specific Flp expression with insertion of *FRT*s in the mouse genome by homologous gene replacement techniques it would be possible to delete or mutate gene segments in restricted cell populations in the transgenic non-human mammal.

In this way, the effect of different mutations can be analyzed in specific cell populations in the background of an otherwise normal animal.

(5) The modularity of these vectors allows them to be readily modified for additional applications such as targeting insertions in the genome or generating chromosomal rearrangements (e.g., insertion, inversion, deletion, duplication, and translocation).

EXAMPLE 2

Materials and Methods

Plasmid Constructions and Production of Transgenic Mice

The *FRT*-disrupted *lacZ* target vector (pFRTZ; Fig. 3A) was generated by inserting the *Hind*III-*Sal*I fragment from pSLhßAPr-lacZ-pA (Zhang et al., 1994) containing human *β-actin* (h*ACTB*) sequences (3-kb 5' flank, 78-bp 5' untranslated region (*UTR*), and 832-bp first intron; Gunning et al., 1987) into the unique *Hind*III and *Sal*I sites of pFRT$_2$neo.lacZ. The control plasmid pFRTZ-product was constructed by inserting the same h*ACTB Hind*III-*Sal*I fragment into pFRT.lacZ. A variant of pFRTZ (designated pFRTZ.2) was generated by inserting the 1.9-kb *Xho*I-*Sal*I fragment from pIC19R-MC1TK (Thomas and Capecchi, 1987) containing the *HSV-thymidine kinase* (*HSV-tk*) gene between the FRT sequences of pFRTZ. Plasmid pNeoßGal (O'Gorman et al., 1991; Snaith et al., 1996; Stratagene) was also used as target DNA. The *FLP* transgene expression vector, phACTB::FLP (Fig. 3B), was constructed by inserting the 3.9-kb *Xba*I-*Sal*I fragment from pSLhßAPr-lacZ-pA into the unique *Xba*I site of pFLP. A non-expressing, negative control *FLP* vector (pRevhACTB::FLP) was constructed which

contains identical h*ACTB* sequences in reverse orientation. To generate pWnt1::FLP, the 2-kb *Sal*I fragment from pFLP, containing a synthetic intron, the sequence encoding Flp (O'Gorman et al., 1991; Stratagene), and SV40 early polyadenylation (pA) sequence, was inserted into the unique *Eco*RV site of pWEXP2 (Echelard et al., 1993). To produce transgenic mice, transgenes were purified away from plasmid sequences and injected into fertilized eggs from B6SJLF$_1$ X B6SJLF$_1$ mice as described by Hogan et al. (1986).

Cell Culture

CCE ES cells (Robertson et al., 1986) were plated onto mitomycin C-treated STO fibroblasts (Robertson, 1987) in Dulbeccos's modified Eagle medium (DMEM) supplemented with 15% fetal bovine serum (FBS), 2 mM glutamine, 0.1 mM ß-mercaptoethanol, 2000 units/ml of leukemia inhibitory factor (LIF; ESGRO, Gibco BRL), 0.1 mM MEM-non essential amino acids, and 30 µM nucleosides. Primary embryonic fibroblasts (EF) were prepared from hemizygous transgenic embryos 13.5 days post coitum (dpc) as described by Robertson (1987). P19 embryonal carcinoma (EC) cells were maintained in a 1:1 mixture of DMEM and Ham F2 medium supplemented with 7.5% FBS and 2 mM glutamine.

Transient Transfection

Transient transfection of ES cells (2x10^5 CCE ES cells in 3.5 cm dishes) was by lipofection (Lipofectamine, Gibco BRL) using 0.5, 2 or 4 µg of plasmid phACTB::FLP (or negative control vector pRevhACTB::FLP), and 0.5 µg of either pFRTZ or pFRTZ-product. ß-Galactosidase (ßGal) activity was detected *in situ* using 4-Cl-5-Br-3-indolyl-ß-d-galactosidase (XGal) (Sanes et al., 1986). Primary EF cultures were plated (5x10^4

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cells/ml) in 3.5 cm dishes and transfected by calcium phosphate precipitation (Gorman et al., 1982) with 3 µg of the target pFRTZ or target pNeoßGal (O'Gorman et al., 1991) followed by XGal stain 48 hours later. P19 EC cells were plated (5x10⁴ cells/ml) in 10 cm dishes. The next day, pairs of duplicate dishes were transfected by calcium phosphate precipitation (Gorman et al., 1982) with 5 µg of target pFRTZ.2 alone, 5 µg of pFRTZ.2 + 5 µg of phACTB::FLP, or 5 µg of pFRTZ.2 + 5 µg of pWnt1::FLP. Twenty-four hours later, half the dishes were treated with either 0.5 µM all-*trans* retinoic acid (RA, Sigma) or control diluent for an additional five days after which cells were stained with XGal.

Detection of Transcripts

Whole mount *in situ* hybridization to 9.5 dpc embryos was performed as described by Wilkinson (1992) using single-strand digoxigenin-UTP labeled RNA probes. The FLP probe (antisense probe 2, Fig. 3B) was a 1386-bp *Eco*RV-*Apa*I fragment from the 3' end of the FLP transgene; control probe (sense) was a 648-bp *Xba*I-*Eco*RV fragment. For Northern analyses, fresh tissue or EF cells were homogenized in 6 M guanidinium isothiocyanate and RNA isolated using acid:phenol (Chomczynski et al., 1987). Total cellular RNA (20 µg) was separated and assayed for hybridization to FLP sequence as described by Sambrook et al. (1989). Ethidium bromide staining of the gel and filter was used to confirm equivalent RNA loading.

Molecular Analysis of Transgenic Mouse Genotypes

Mouse tails were lysed with NaDodSO₄/proteinase K and treated with phenol/chloroform, 1:1 (vol/vol), precipitated with ethanol, and dissolved in 10 mM Tris·HCL, pH 8 and 1 mM EDTA. For PCR analysis, DNAs were amplified with the following primers: SD42 (SEQ ID NO:20) and SD41 (SEQ ID NO:21), for the *FLP*

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transgene (0.75-kb amplified fragment); SD49 (SEQ ID NO:22) and SD68 (SEQ ID

NO:23), for the *FRTZ* transgene (1.4-kb amplified fragment) and *FRTZ-product* (0.25-kb

fragment). The 0.25-kb PCR amplification product was cloned into plasmid pCR

(Invitrogen) and sequenced; sequence analysis of the 0.25-kb product showed precise site-

specific recombination. Genomic DNA isolated from freshly harvested tissues (Burrell,

1995) was subjected to *Bam*HI/*Sca*I digestion, and Southern blot analyses. Radiolabeled

DNA fragments (specific activity of $2-5 \times 10^8$ cpm/µg) for use as probes were prepared by

random priming (Feinberg and Vogelstein, 1993). Transgene copy number was estimated

by including standard amounts of the injected transgene in parallel. Quantitation of

radioactivity in specific bands was performed with a Molecular Dynamics

PhosphorImager.

Strategy Used to Assay Flp Function in Cell Culture and the Mouse

To generate a test recombination substrate for Flp function, a *lacZ* gene was

disrupted by inserting an *FRT* cassette that contains stop codons in all three reading

frames. This target transgene is referred to as *FRTZ*, for *FRT*-disrupted *lacZ* (Fig. 3A).

Because the two *FRT* sequences flanking the cassette are in the same orientation, Flp

activity should excise the intervening DNA leaving a single residual *FRT* in frame with

lacZ (Fig. 3C). Because there are no ATG codons to initiate translation of functional ßGal

downstream of the *FRT*-cassette, ßGal activity is strictly dependent on Flp-mediated

excisional recombination in a manner similar to previously described gain-of-function

systems (O'Gorman et al., 1991; Struhl and Basler, 1993).

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To broadly express both *FLP* and the *FRT*-disrupted *lacZ* target (*FRTZ*), both

transgenes were placed under the control of regulatory sequences from the human *ß-actin*

(h*ACTB*) gene (Fig. 3A, B). These h*ACTB* sequences have been shown to be active in

most tissues in transgenic mice (Zhang et al., 1994). A "recombined" control transgene,

FRTZ-product, representing the predicted product of Flp recombination was also

constructed (Fig. 3A).

Flp-Mediates Efficient Recombination of Extrachromosomal DNA in ES Cells

The efficacy of Flp-mediated excisional recombination of extrachromosomal DNA

in ES cells was tested by assaying for gain of ßGal activity following transient

cotransfection with target and recombinase plasmids. Cells were transiently transfected

with either pFRTZ plus phACTB::FLP, or pFRTZ plus the negative control plasmid

pRevhACTB::FLP, followed by XGal stain 48 hours later. Positive control cultures were

transfected with the "recombined" plasmid, pFRTZ-product. Cultures transfected with

target plasmid pFRTZ, alone or with pRevhACTB::FLP, showed no detectable ßGal

activity; in contrast, robust activity was observed following cotransfection with

phACTB::FLP.

To estimate recombinase activity, XGal-positive cells in each transfection were

counted and compared. The number of cells staining blue after transfection with the

control "recombined" pFRTZ-product reflected transfection efficiency and, since

constitutively active, the maximal number of ßGal-positive cells. Cotransfection with a

fixed amount of target plasmid and increasing amounts of *FLP* expression vector resulted

in an increasing percentage of XGal-positive cells relative to control pFRTZ-product

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transfections. A comparison between experimental (pFRTZ + phACTB::FLP) and control (pFRTZ-product) transfections showed that Flp-mediated ßGal activation occurred in at least 30% of transfected ES cells and could be as high as 78%. This increase in recombination with increasing Flp-encoding plasmid likely reflects more Flp protein produced per cell, as well as an increase in the proportion of cells that took up both the target and Flp-encoding plasmids (and thereby had the potential to activate *lacZ*).

Flp Can Be Generally Expressed in the Mouse Without Deleterious Effects

To determine whether Flp can function in the mouse and whether Flp expression, itself, would have any adverse effects, mice carrying the h*ACTB::FLP* transgene were generated. To identify mouse lines producing Flp in a wide range of tissues, F1 mice from each founder (4917, 4924, 4925, 4927, 4921) were screened for ubiquitous *FLP* mRNA and recombinase activity. The distribution and amount of *FLP* mRNA was assessed in the embryo by whole mount *in situ* hybridization using antisense probe 2 (Fig. 3B) and a control sense probe, and in adult tissues by Northern analysis using hybridization to ^{32}P-labeled probe 3 (Fig. 3B). Two of the five h*ACTB::FLP* mouse lines exhibited broad patterns of *FLP* transcripts in 9.5 dpc hemizygous embryos (mouse lines 4917 and 4924) and in adult tissues (e.g., testes, brain, heart, intestine, kidney, lung, ovary, striated muscle). Line 4924 expressed in the liver, but liver expression for line 4917 was weak; line 4917 expressed in the spleen and uterus, but there was only variable expression in those organs for line 4924.

Flp recombinase activity was assayed in embryonic fibroblast (EF) cultures derived from each transgenic mouse line. Primary EF cultures were prepared from hemizygous

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h*ACTB::FLP* transgenic embryos as described by Robertson (1987), transiently transfected with 3 μg of target pNEOβ-GAL plasmid (O'Gorman et al., 1991), and followed 48 hours later by histochemical Xgal staining (Sanes et al., 1986). As indicated by the number of blue cells, maximal Flp activity (approximately 45% of the "recombined" control) was observed in lines 4917 and 4924, the same mouse lines that showed broad *FLP* expression. The amount of recombinase activity detected in EF cultures also correlated with the amount of *FLP* mRNA isolated from each culture. From these results, it can be inferred that mouse lines 4917 and 4924 are the best candidates for broadly expressed active recombinase. Because no abnormalities were detected in founders or offspring, it is likely that Flp activity is nontoxic and can be used in most cell types.

Flp is Necessary and Sufficient to Recombine Target Sequences in Transgenic Mice

To test whether Flp activity can recombine a chromosomal target *in vivo*, mice carrying the *FRT*-disrupted *lacZ* transgene, *FRTZ*, were generated. Five transgenic founders were obtained. F_1 mice from four of the five founders bred as expected for unique single-site integration events (one founder failed to transmit the transgene). Southern analysis of liver DNA isolated from each mouse line showed that three of the four mouse lines carried the target *FRT*-disrupted *lacZ* transgene in head-to-tail array: line 4999 carried an array of approximately 4 copies of the *FRTZ* transgene, line 4998 had 11 copies, and line 5000 had 30 copies. Transgene transmission was mendelian and no rearrangements were observed.

The ability of Flp to catalyze *in vivo* recombination of the target *FRTZ* transgene was initially examined by crossing these mouse lines with the Flp producing lines

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described above (4917 and 4924). Tail DNA from doubly transgenic animals was analyzed by PCR using primers (diagrammed in Fig. 3A,B) specific for detecting either the *FRTZ* transgene, the recombined target *FRTZ-product*, or the *FLP* transgene. Analyses of progeny from three distinct crosses are show the product of Flp-mediated excisional recombination at the *FRTZ* locus was amplified only in DNA isolated from doubly transgenic mice and was not detected in littermates transgenic for only the recombinase or the target gene. All three *FRTZ* target lines were found to be competent for recombination by this assay. Sequence analysis of the 0.25-kb amplification product showed precise site-specific recombination.

Flp Mediates Recombination in a Variety of Tissues in a Dose-Dependent Manner

The efficiency of Flp recombination at target *FRTZ* loci was assayed by Southern analysis. Genomic DNA isolated from doubly transgenic adult mice (target line *FRTZ-4999; FLP-4917*) was hybridized with a *lacZ* probe (probe 1 in Fig. 3A) to allow simultaneous detection of the target *FRTZ* transgene and the product of recombination. The new 4.4-kb DNA fragment resulting from the recombined target was present only in samples from doubly transgenic animals, and absent in DNA isolated from either target *FRTZ* or *FLP* littermates.

The amount of recombination product detected by Southern analysis was found to correlate directly with the amount of *FLP* mRNA detected in each tissue by Northern hybridization examined (e.g., liver, muscle, testes). Estimates of recombination efficiency were obtained from phosphorimage quantification of recombined (4.4-kb) to non-recombined (5.6-kb) bands. In muscle, approximately 30% of the transgenes were in the

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recombined (4.4-kb) configuration. This represents an average of the actual recombination achieved in the various cell types isolated when dissecting muscle tissue (myofibrils, connective tissue fibroblasts, vascular endothelial cells, lymph node cells, blood cells). The value of 30% therefore represents a low estimate of the maximal recombination efficiency. This frequency is consistent with that observed in the EF cell culture assay derived from the same *FLP-4917* mouse line (45%); indeed, both cell populations showed similar amounts of *FLP* mRNA. Hybridizing with a probe specific to DNA between the *FRT* sites detected only the unrecombined fragment.

The Recombined Transgene Is Stably Transmitted Through the Germline

A prerequisite to using Flp to genetically manipulate cell lineages is that the recombination product be stable and heritable. Germline transmission of the recombined transgene was demonstrated by outcrossing a doubly transgenic (*FRTZ-5000; FLP-4917*) male and genotyping progeny by PCR. Both recombined and unrecombined transgenes were detected in this singly transgenic F_3 mouse indicating that recombination was incomplete; a subset of the 30 *FRTZ* transgenes in tandem array underwent recombination.

Conditional Expression of Flp Can Induce Regulated Rearrangement of Target Sequences in Differentiating EC Cells

Controlling expression of the *FLP* transgene is a way to restrict recombination, and therefore gene activation or inactivation, to a specific cell population (e.g., those cells expressing sufficient Flp activity to catalyze FRT site-specific recombination). Whether Flp-mediated recombination could be induced in a differentiating EC cell culture system

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by using *Wnt-1* regulatory sequences (Echelard et al., 1994) to express *FLP* (see Fig. 3B for *Wnt1::FLP* transgene) was investigated. Retinoic acid (RA) can induce pluripotent P19 EC cells to differentiate into a mixed population of fibroblasts, astrocytes, and neural cells (Rudnicki and McBurney, 1987; Schuuring et al., 1989). In this manner, *Wnt-1* expression is likely induced specifically in neural derivatives, paralleling that seen in embryos where *Wnt-1* mRNA is detected in differentiating neuroectoderm (Wilkinson et al., 1987).

P19 cells were transiently transfected with target plasmid, target plus phACTB::FLP, or target plus pWnt1::FLP; 0.5 µM RA or control diluent was added to the monolayer twenty four hours later. Following five days of RA treatment, ßGal activity was assessed by histochemical XGal staining. Neural induction was monitored by morphology (the presence of long cellular processes) and culture senescence, as well as by induction of endogenous *Wnt-1* mRNA.

ßGal activity was detected in target plus pWnt1::FLP cotransfections only following RA induced differentiation. Similarly, endogenous *Wnt-1* expression was absolutely dependent on RA. Low levels of *Wnt-1* transcripts were first detected by Northern hybridization after four days of RA treatment; no *Wnt-1* RNA was detected in untreated cells. As predicted by the nature of the h*ACTB* regulatory sequences, ßGal-positive cells were observed in the target plus phACTB::FLP cotransfections independent of RA. The target plasmid alone showed no activity. In addition to demonstrating regulated rearrangement of target sequences, these results define a temporal relationship between *FLP* expression and completed recombination. ßGal activity, reflecting Flp-recombination, was observed in the target plus pWnt1::FLP cotransfection within 24 hours of first detecting *Wnt-1* transcripts, and by inference *Wnt1::FLP* mRNA.

Conclusions

The present example demonstrates that Flp can effectively recombine extrachromosmal target DNA in ES cells, EC cells and chromosomal target DNA in transgenic mice. Flp can direct site-specific and heritable DNA recombination in the mouse, and regulated (inducible) recombination in differentiating EC cells. These properties indicate that Flp can be used to make directed modifications of a transgenic non-human mammalian genome.

Using the Flp-FRT system, recombination of an extrachromosomal target can occur in ES cells with an efficiency similar to that previously observed in mouse embryonal carcinoma (F9) cells (O'Gorman et al., 1991) and in monkey (CV-1) and human (293) embryonic kidney cells (O'Gorman et al., 1991; Logie and Stewart, 1994). Because the efficiency of Flp recombination on an extrachromosomal substrate estimated here (30-78%) is comparable to that reported for Cre on a chromosomal substrate (40-80%; Gu et al., 1993), the Flp-FRT system can likely be exploited to similarly manipulate ES cell chromosomal DNA. Towards this end, a more elaborate two-step selection scheme was employed where Flp-mediated deletion of an integrated selectable marker gene (*PGK-neo*) was reported to occur in 90% of Flp-expressing ES cells (Fiering et al., 1995).

In the mouse, the present example shows Flp expression is necessary and sufficient for excisional recombination of *FRT* target sequences. Because recombination was detected at all three chromosomal sites assayed, it is likely that most chromosomal transgenes will be accessible to Flp function. The extent of recombination observed in a given tissue correlated directly with the overall amount of *FLP* mRNA detected in that tissue; it is important to note that this type of tissue analysis presents an average and

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therefore may underestimate the maximal recombination achieved in a specific cell type. Nonetheless, these results define a dose-effect relationship which suggests that different degrees of recombination (frequent or rare) can be attained by varying the strength and specificity of the sequences used to express *FLP*. For some experiments, complete (quantitative) recombination may be needed. The results presented here suggest that one means to achieve this is to increase the level of *FLP* expression. Alternative strategies include identifying Flp variants with higher activity in mammalian cells, or to enhance the nuclear localization of Flp.

The finding that Flp can be generally expressed in the mouse without adverse effects suggests that Flp-recombination between random sequences in the mouse genome is rare. If high levels of illegitimate (non-*FRT*) recombination were occuring due to Flp expression, abnormalities would be expected in *FLP* founders or offspring. No adverse effects were detected. This result suggests that Flp can be used to mediate recombination in a variety of cell types.

Flp-mediated excisional recombination is sufficiently dose sensitive that recombination can be regulated in differentiating EC cells in culture. This was evident from examination of RA-treated P19 cells in which the *Wnt-1* promoter was used to express *FLP*. The temporal induction of *Wnt-1* transcripts following RA-induced differentiation indicates that recombination occurred relatively quickly: *FLP* expression, recombination of the target transgene to reconstitute a functional *lacZ* gene, and subsequent ßGal production occurred within twenty four hours. These results demonstrate that regulated rearrangement of a target sequence can be achieved.

The demonstration that Flp can excise DNA in mice and that the recombination product is heritable, suggests that Flp will be useful to study cell lineages. Considering this potential application, the initial test recombination substrate was designed to indicate and "remember" a recombination event by the irreversible gain of ßGal activity (dependent only on constitutive promoter activity). Mice transgenic for this target should have the capability of marking cell lineages following introduction of Flp by crossing. Towards this end, mice transgenic for *Wnt1::FLP* have been generated; by crossing to an "optimal" target mouse, cells originating from the dorsal aspect of the developing central nervous system are predicted to be marked. Although all three *FRTZ* target lines analyzed here were competent for recombination, none of the recombined target alleles were sufficiently active to allow cell marking by XGal stain. The lack of ßGal activity associated with the observed recombination most likely reflects a position effect on transgene transcription exerted by the genomic integration site since only one in four control *FRTZ-product* mouse lines expresses ßGal. Such sensitivity to chromosomal context is also supported by the variation in transcript profiles observed when using the same h*ACTB* regulatory sequences to direct *FLP* expression (two of five lines showed general expression). Importantly, by screening additional *FRTZ* target loci, a chromosomal integration site has been identified that can support *lacZ* expression following Flp-recombination.

These examples demonstrate that Flp can serve as a tool to alter a mammalian genome *in vivo*. By employing Flp and another recombinase (e.g., Cre and site-specific recombinases cited in U.S. Pat. No. 5,434,066), it should be possible to engineer multiple recombination reactions (multiple gene activation or inactivation events) in a transgenic non-human mammal.

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While the present invention has been described in connection with what is presently considered to be practical and preferred embodiments, it is understood that the present invention is not to be limited or restricted to the disclosed embodiments but, on the contrary, is intended to cover various modifications and equivalent arrangements included within the spirit and scope of the appended claims.

Thus, it is to be understood that variations in the described invention will be obvious to those skilled in the art without departing from the novel aspects of the present invention and such variations are intended to come within the scope of the claims below. In particular, although the claimed invention is described in terms of the Flp recombinase, wild type Flp and the Flp-F70L mutant protein (SEQ ID NO:17), and the Flp-recognition sequence (e.g., FRT such as, for example, SEQ ID NOS:14 and 15), one skilled in the art would understand that functional equivalents of Flp recombinase and Flp-recognition sequences could be used in the context of the claimed invention and are intended to come within the scope of the claims below.